per immediate container that it is represented to contain.

(2) *Powder packaged with inert gases.* Spray, as directed in the labeling, the entire contents of each container to be tested into a separate 2-liter Erlenmeyer flask, held in a horizontal position. Add 500 milliliters of 0.1 *N* HCl and shake to dissolve the contents. Immediately remove aliquots of this solution and, using 0.1 *M* potassium phosphate buffer, pH 4.5, for further dilutions, proceed as directed in §446.10a(b)(1) of this chapter if it is chlortetracycline hydrochloride powder or §446.81a(b)(1) of this chapter if it is tetracycline hydrochloride powder. Calculate the average total amount of antibiotic expelled from the containers. The total potency is satisfactory if it contains not less than 85 percent of the number of milligrams of chlortetracycline hydrochloride or tetracycline hydrochloride that it is represented to contain.

(b) *Moisture.* Proceed as directed in §440.80a(b)(5)(i) of this chapter, except if it is packaged with inert gases proceed as directed in §536.513(c) of this chapter.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§436.515 Capsules tetracycline and oleandomycin phosphate; capsules tetracycline and troleandomycin; capsules tetracycline hydrochloride and oleandomycin phosphate; capsules tetracycline hydrochloride and troleandomycin.

(a) *Potency*—(1) *Tetracycline or tetracycline hydrochloride content by turbidimetric assay*—(i) *Test culture and media.* Maintain the test organism *Escherichia coli* (ATCC 10536)[1] on the agar described in §440.80a(b)(1) (ii)(*a*) of this chapter. For use in the assay, prepare a suspension of the organism every 2 weeks, as follows: Transfer the organism to a fresh agar slant and incubate at 37°C. overnight. Wash the growth from the slant with the aid of 2 milliliters of sterile distilled water and sterile glass beads into a Roux bottle containing 300 milliliters of the maintenance medium. Incubate overnight at 37° C. and then harvest the growth with 50 milliliters of sterile distilled water and sterile glass beads. Standardize this suspension by determining the dilution that will permit 40-percent light transmission in a photoelectric colorimeter using a 650-millimicron filter and an 18-millimeter diameter test tube as an absorption cell. Prepare the daily inoculum by adding 10 milliliters of that dilution to each liter of nutrient broth, prepared as directed in §440.80a (b)(1)(ii)(*c*) of this chapter, needed for the test.

(ii) *Working standard and solutions.* Dissolve an appropriate amount of the working standard in sufficient 0.1 *N* HCl to give a concentration of 1,000 micrograms per milliliter. This stock solution may be kept in the refrigerator for 1 week. Make daily dilutions of the stock solution with 0.1 *M* potassium phosphate buffer (pH 4.5) to obtain concentrations of 0.146, 0.187, 0.240, 0.308, and 0.395 micrograms per milliliter. Add 1.0 milliliter of each such concentration to each of three 16 millimeters x 125 millimeters test tubes.

(iii) *Preparation of sample.* Dissolve the contents of a representative number of capsules in sufficient 0.1 *N* HCl to give a stock solution of convenient concentration. Further dilute the stock solution with 0.1 *M* potassium phosphate buffer (pH 4.5) to obtain a final concentration of 0.24 microgram per milliliter (estimated). Add 1.0-milliliter of this dilution to each of three 16 millimeters x 125 millimeters test tubes.

(iv) *Procedure.* To each of the 16 millimeters x 125 millimeters test tubes prepared in paragraph (a)(1)(ii) and (iii) of this section, add 9.0 milliliters of the inoculated nutrient broth described in paragraph (a)(1)(i) of this section and place immediately in a 37° C. water bath for 3 to 4 hours. After incubation, add 0.5 milliliter of a 12-percent formaldehyde solution to each tube and read the absorbance values in a suitable photoelectric colorimeter using a wavelength of 530 millimicrons. Set the instrument at zero absorbance with clear uninoculated broth prepared as described in §440.80a(b)(1)(ii)(*c*) of this chapter.

(v) *Estimation of potency.* Plot the average values for each concentration of

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

the standard on arithmetic graph paper with absorbance values on the ordinate and tetracycline or tetracycline hydrochloride concentrations on the abscissa. Construct the best straightline through the points, either by inspection or by means of the following equations:

$$L=(3a+2b+c-e)/5,$$
$$H=(3e+2d+c-a)/5,$$

where:

L=absorbance value for the lowest concentration of the standard curve.

H=absorbance value for the highest concentration of the standard curve.

a, b, c, d, e=average absorbance values for each concentration of the standard curve.

Plot the values obtained for *L* and *H* and connect the points with a straight line. Average the absorbance values for the sample and read the tetracycline or tetracycline hydrochloride concentration from the standard curve. Multiply the concentration by appropriate dilution factors to obtain the tetracycline or tetracycline hydrochloride content of the sample. Its potency is satisfactory if it contains the equivalent of not less than 85 percent of the number of milligrams of tetracycline hydrochloride that it is represented to contain.

(2) *Oleandomycin content.* (i) If oleandomycin phosphate is used, proceed as directed in paragraph (c)(1) of this section, except prepare the sample as follows: Dissolve the contents of a representative number of capsules in sufficient 0.1 *M* potassium phosphate buffer (pH 8.0) to give a stock solution of convenient concentration. Further dilute with 0.1 *M* potassium phosphate buffer (pH 8.0) to obtain a final concentration of 5.0 µg. of oleandomycin activity per milliliter (estimated).

(ii) If troleandomycin is used, proceed as follows: Dissolve the contents of a representative number of capsules in chloroform to give a stock solution of 1.0 milligram of oleandomycin activity per milliliter. Transfer 30 milliliters of the chloroform solution to a glass-stoppered test tube (200 millimeters x 22 millimeters) and add 20 milliliters of 1 *N* sodium hydroxide. Shake for 1 minute and centrifuge briefly to aid in the separation of the layers. With the aid of a syringe and needle, remove and discard the aqueous layer. Repeat the washing procedure with two more 20-milliter portions of 1 *N* sodium hydroxide solution. Filter the chloroform layer through a pledget of cotton. Dilute an aliquot of this solution with chloroform to give a solution containing approximately 25 µg. of oleandomycin per milliliter. Transfer a 5.0 milliliter aliquot to a 40 milliliter glass-stoppered centrifuge tube, dilute to 20 milliliters, with chloroform, and determine the oleandomycin content as directed in paragraph (d)(1)(i) of this section.

Its content of oleandomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 440.80a(b)(5)(1) of this chapter.

(c) *Oleandomycin phosphate used in making the capsules*—(1) *Potency*—(i) *Cylinders* (*cups*). Used cylinders described in § 440.80a(b)(1)(i) of this chapter.

(ii) *Culture media.* (*a*) Use the nutrient agar described in § 440.80a (b)(1)(ii)(*a*) of this chapter for the seed layer and base layer, except that its pH after sterilization is 7.8 to 8.0.

(*b*) Use the nutrient agar described in § 440.80a(b)(1)(ii)(*a*) of this chapter for maintaining the test organism.

(iii) *Working standard.* Dissolve a suitable weighed quantity (usually 25 milligrams or less) of the working standard (obtained from the Food and Drug Administration) in 2 milliliters of ethanol, then add sufficient 0.1 *M* potassium phosphate buffer, pH 8.0, to give a concentration of 1,000 micrograms of oleandomycin base per milliliter. This stock solution may be kept in the refrigerator for 3 days.

(iv) *Preparation of sample.* Dissolve the sample in sufficient 0.1 *M* potassium phosphate buffer (pH 8.0) to give a convenient stock solution. Further dilute in 0.1 *M* potassium phosphate buffer (pH 8.0) to give a final concentration of 5.0 micrograms per milliliter (estimated).

(v) *Preparation of test organism.* The test organism is *Staphylococcus*

epidermidis (ATCC 12228)[1] which is maintained on slants or agar described under paragraph (c)(1)(ii)(*a*) of this section. Wash the organism from the agar slant with 3 milliliters of sterile physiological saline solution onto a large agar surface such as that provided by a Roux bottle containing 300 milliliters of the agar described in paragraph (c)(1)(ii)(*a*) of this section. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate for 4 hours at 32° C. and then wash the resulting growth from the agar surface with about 30 milliliters of sterile physiological saline solution. Standardize the suspension by determining the dilution that will give 80-percent light transmission, using a suitable photoelectric colorimeter with a 650-millimicron filter and an 18-millimeter-diameter test tube as an absorption cell. Run test plates to determine the quantity of the diluted suspension (usually 1.5 milliliters) that should be added to each 100 milliliters of agar to give clear, sharp zones of inhibition of appropriate size.

(vi) *Preparation of plates.* Add 21 milliliters of the agar prepared as described in paragraph (c)(1)(ii)(*a*) of this section to each Petri dish (20 millimeters × 100 millimeters). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared. Melt a sufficient amount of the agar described in paragraph (c)(1)(ii)(*a*) of this section, cool to 48° C., add the proper amount of the test organism as described in paragraph (c)(1)(v) of this section and mix thoroughly. Add 4 milliliters of this inoculated agar to each Petri dish. Distribute the agar evenly in the plates, cover with porcelain covers glazed on the outside, and allow to harden. After the agar has hardened, place 6 cylinders on the agar surface so that they are at approximately 60° intervals on a 2.8-centimeter radius.

(vii) *Standard curve.* Prepare the daily standard curve by further diluting the 1,000 micrograms per milliliter stock solution in 0.1 *M* potassium phosphate buffer (pH 8.0) to obtain concentrations of 3.2, 4.0, 5.0, 6.25 and 7.80 micrograms per milliliter. Use 3 plates for the determination of each point on the curve, except the 5.0 micrograms per milliliter concentration, a total of 12 plates. On each of 3 plates fill 3 cylinders with the 5.0 micrograms per milliliter standard, and the other 3 cylinders with the concentration under test. Thus, there will be 36 five-microgram determinations and 9 determinations for each of the other points on the curve. After incubation, read the diameters of the circles of inhibition in the plates. Average the readings of the 5.0 micrograms per milliliter concentration and the readings of the point tested for each set of 3 plates and average also all 36 readings of the 5.0 micrograms per milliliter concentration. The average of the 36 readings of the 5.0 micrograms per milliliter concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 5.0 micrograms per milliliter reading for that set of 3 plates were the same as the correction point. Thus, if in correcting the 4.0-microgram concentration, the average of the 36 readings of the 5.0-microgram concentration were 20.0 millimeters, and the average of the 5.0-microgram concentration of this set of 3 plates were 19.8 millimeters, the correction would be +0.2 millimeter. If the average reading of the 4.0-microgram concentration of these same 3 plates were 19.0 millimeters, the corrected value would be 19.2 millimeters. Plot these corrected values, including the average of the 5.0 micrograms per milliliter concentration, on 2-cycle semilog paper, using the concentration in micrograms per milliliter as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points, either by inspection or by means of the following equations:

$$L=(3a+2b+c-e)/5,$$
$$H=(3e+2d+c-a)/5,$$

where:

L=corrected zone diameter for the lowest concentration of the standard curve,

H=corrected zone diameter for the highest concentration of the standard curve,

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

c=average zone diameter for 36 readings of the 5.0 micrograms per milliliter standard.

a, b, d, e=corrected average values for the 3.2, 4.0, 6.25, and 7.81 micrograms per milliliter standard solutions, respectively.

Plot the values obtained for *L* and *H* and connect with a straight line.

(viii) *Assay.* Use 3 plates for each sample. Fill 3 cylinders on each plate with the standard 5.0 micrograms per milliliter solution and 3 cylinders with the 5.0 micrograms per milliliter (estimated) sample, alternating standard and sample. Incubate all plates, including those containing the standard curve, at 32° C.–35° C. overnight, and measure the diameter of each circle of inhibition. To estimate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on the 3 plates used. If the sample gives a larger zone size than the average of the standard, add the difference between them to the 5.0 micrograms per milliliter zone on the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 5.0 micrograms per milliliter value on the curve. From the standard curve, read the potencies corresponding to these corrected values of zone sizes.

(2) *Toxicity.* Proceed as directed in § 440.80a(b)(4) of this chapter, except use physiological salt solution as the diluent, and inject 0.5 milliliter of a solution containing 8 milligrams per milliliter.

(3) *Moisture.* Proceed as directed in § 440.80(b)(5)(i) of this chapter.

(4) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a solution containing 100 milligrams per milliliter.

(5) *Crystallinity.* Proceed as directed in § 440.80a(b)(5)(iii) of this chapter.

(d) *Troleandomycin used in making the capsules*—(1) *Potency*—(i) *Chemical method*—(*a*) *Reagents and equipment.* (*1*) Methyl orange reagent: Shake 0.5 *M* boric acid solution for about 12 hours (to insure saturation) with an excess of methyl orange indicators. An alternative method is to heat the mixture to about 50° C. and shake for about an hour. Then allow to cool. Filter the saturated dye solution and wash three times with chloroform. Store the dye solution over chloroform.

(*2*) Acid-alcohol solution: Add 2 milliliters of concentrated sulfuric acid to 98 milliliters of absolute methyl alcohol.

(*3*) Glycerin: Reagent grade.

(*4*) Centrifuge tubes: 40 milliliters, glass-stoppered.

(*b*) *Procedure.* Prepare a chloroform solution containing 50.0 milligrams activity of standard oleandomycin base in 200 milliliters of solution. Transfer 10.0 milliliters of the solution to a 100-milliliter volumetric flask and dilute to volume with chloroform. Transfer 2.0, 4.0, 6.0, and 8.0 milliliters of this solution to glass-stoppered centrifuge tubes (40-milliliter size) and dilute to a total volume of 20.0 milliliters each with chloroform. To the 20.0 milliliters of the solution present in each (40-milliliter size) centrifuge tube add 0.2 milliliter of glacial acetic acid, 0.20 milliliter of glycerin, and 0.40 milliliter of methyl orange reagent. Shake for 5 minutes and centrifuge for 3 minutes. Immediately transfer to another tube a 10.0-milliliter aliquot from the chloroform (lower) layer. Care must be exercised to see that no portion of the dye-glycerin-phase is included with the chloroform aliquot. Add 1.0 milliliter of acid-alcohol solution to this chloroform aliquot, mix well, and read the absorbancy at 535 mμ, using a 1-centimeter cell and a suitable photometer and using chloroform, similarly treated, as a blank. Prepare a standard curve, plotting the absorbance values of the standard solutions against the concentration expressed in micrograms per aliquot. Accurately weigh the sample to be tested to give 50 milligrams (estimated) of oleandomycin activity, dissolve in chloroform, and make to 200 milliliters with chloroform. Transfer 10.0 milliliters to a 100-milliliter volumetric flask and make to volume with chloroform. Transfer 5.0 milliliters to a glass-stoppered centrifuge tube and proceed as above. Determine the potency of the sample from the standard curve.

(ii) *Microbiological assay.* Proceed as directed in paragraph (c)(1) of this section, except:

(*a*) In lieu of the directions in paragraph (c)(1)(ii)(*a*) of this section, use

the nutrient agar described in § 440.80a(b)(1)(ii)(*a*) of this chapter for the seed and base layers, except add 2.0 milliliters of polysorbate 80 to each 100 milliliters of agar. Its pH after sterilization is 7.8 to 8.0.

(*b*) In lieu of the directions in paragraph (c)(1)(iii) of this section, dissolve a suitable weighed quantity (usually 25 milligrams or less) of the troleandomycin working standard (obtained from the Food and Drug Administration) in sufficient 80 percent isopropyl alcohol-water solution to give a concentration of 1,000 micrograms per milliliter (estimated). Use the solution the day that it is prepared.

(*c*) In lieu of the directions in paragraph (c)(1)(iv) of this section, dissolve the sample in sufficient 80 percent isopropyl alcohol-water solution to give a convenient stock solution. Further dilute in 0.2 *M* potassium phosphate buffer, pH 10.5 (35 grams of dipotassium phosphate plus 2 milliliters of 10 *N* NaOH, q.s. to 1 liter), to give a final concentration of 15 micrograms per milliliter (estimated).

(*d*) In lieu of the directions in paragraph (c)(1)(vi) of this section, use the agar described in paragraph (d)(1)(ii)(*a*) of this section for both layers. Use the plates as soon after seeding as is practical. If they are not to be used shortly after seeding, then they should be refrigerated until ready for use.

(*e*) In lieu of the directions for preparing the standard curve in paragraph (c)(1)(vii) of this section, prepare the standard curve by diluting the stock solution in 0.2 *M* potassium phosphate buffer, pH 10.5, to give concentrations of 9.6, 12.0, 15.0, 18.8, and 23.4 micrograms per milliliter. The 15.0 micrograms per milliliter is the reference concentration.

(*f*) In lieu of the directions in paragraph (c)(1)(viii) of this section, incubate the plates at 37° C. overnight. The concentration of the sample and standard being tested is 15.0 micrograms per milliliter.

(2) *Toxicity.* Administer orally, by means of a cannula or other suitable device, to each of five mice within the weight range of 18 grams to 25 grams, 0.5 milliliter of a suspension containing 200 milligrams per milliliter in normal saline solution. If no animal dies within 48 hours, the sample is nontoxic. If one or more animals die within 48 hours, repeat the test, using for each test five or more previously unused mice weighing 20 grams (±0.5 gram) each; if the total deaths within 48 hours is no greater than 10 percent of the total number of animals tested, including the original test, the sample is nontoxic.

(3) *Moisture.* Proceed as directed in § 440.80a(b)(5)(i) of this chapter.

(4) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a saturated aqueous-ethanol (1:1) solution prepared by adding 100 milligrams per milliliter.

(5) *Paper chromatograph method*—(i) *Apparatus and reagents*—(*a*) Chromatographic chamber (cylinder glass-stoppered museum jar 11.5 inches × 3.5 inches).

(*b*) Chromatographic paper (8 inches × 8 inches Whatman No. 1).

(*c*) 0.1 *N* hydrochloric acid.

(*d*) Resolving solvent: Butyl acetate, benzene, nitromethane, pyridine (5:5:5:1 by volume).

(*e*) Spray reagent: 15 grams antimony trichloride per 100 milliliters of chloroform.

(ii) *Procedure.* Dissolve the sample in chloroform to give a solution containing 10 milligrams to 20 milligrams per milliliter. Prepare a sheet of chromatographic paper by drawing a line of origin parallel to and 1 inch from the edge of the paper. Wet the paper thoroughly with the 0.1 *N* hydrochloric acid and blot it firmly between sheets of absorbent paper. Starting 2 inches in from the edges and at 1-inch intervals, apply 3 microliters to 5 microliters of the sample solutions to the starting line. Allow a few minutes for the paper to dry partially. While the paper is still damp, form a cylinder by bringing the outer edges together, allowing about 1-inch overlap, and secure with a paper clip. Stand the paper in the chromatographic chamber, which has been filled to a depth of ½-inch with the resolving solvent. After the solvent front rises to a height of 4 inches to 5 inches above the origin, remove the paper from the tank and hang it up to air dry. Spray the dried paper with the antimony trichloride reagent. Hang the paper in a 100° C. oven for 3

minutes. A purple spot becomes visible for trioleandomycin at an R_f value of about 0.85. The approximate R_f values for diacetyloleandomycin, monoacetyloleandomycin, and oleandomycin are, respectively, 0.72, 0.27, and 0.13.

(6) *Acetyl determination*—(i) *Apparatus and reagents.* (*a*) One three-necked Pyrex flask of approximately 45 milliliters capacity, pear-shaped with T-joints, agar inlet tube, glass-stoppered funnel, glass condenser, and bubble counter.

(*b*) 50-milliliter Pyrex Erlenmeyer flask.

(*c*) 10-milliliter burette, calibrated in 0.02 milliliter.

(*d*) Anhydrous methanol, reagent grade.

(*e*) 2 *N* sodium hydroxide solution.

(*f*) Sulfuric acid solution prepared by adding 100 milliliters of concentrated H_2SO_4 to 200 milliliters of water.

(*g*) 1 *N* barium chloride solution.

(*h*) Phenolphthalein solution (1 percent in ethanol).

(*i*) Water-pumped nitrogen.

(*j*) NaOH solution, 0.015 *N*.

(ii) *Procedure.* Weight accurately (to 0.01 milligram) approximately 30 milligrams of the sample into the three-necked acetyl flask. Add 2.0 milliliters of methanol to dissolve the sample, then add slowly with gentle swirling, 1.0 milliliter of NaOH solution. Connect the gas inlet tube with bubble counter attached, and adjust nitrogen flow to about two bubbles a second. Put glass-stoppered funnel in centerneck of acetyl flask and put about 5 milliliters of H_2O in the funnel. Add a boiling chip to the solution and attach condenser in the refluxing position with water cooling. Adjust burner flame under acetyl flask to reflux solution gently. Reflux for 30 minutes. Cool assembly slightly then rinse down condenser (still in reflux position) with a few milliliters of H_2O. Reassemble condenser to the distillation position and add water through the funnel to make a total of approximately 5 milliliters of H_2O added to acetyl flask. Adjust burner flame so that about 5 milliliters of H_2O and methanol is distilled over in approximately 10 minutes. Discard this distillate. Cool acetyl flask slightly. Acidify solution in flask by adding 1 milliliter of the sulfuric acid solution through the funnel. Adjust burner flame and distill over approximately 20 milliliters of distillate into an Erlenmeyer flask in about 20 minutes, adding water through the funnel as necessary. It is important to keep the liquid volume in the acetyl flask around 2 milliliters to 3 milliliters in order to obtain a quantitative recovery of the acetic acid. Collect a second fraction of distillate, about 10 milliliters in volume. As the second fraction is distilling, process the first fraction. Heat the first reaction and boil gently about 20 seconds. Add a few drops of $BaCL_2$ solution to check if any sulfate was distilled over. If the sulfate is present, discard and repeat the whole determination. If the sulfate is absent immediately titrate the solution with the 0.015 *N* NaOH solution to a faint pink endpoint, using one drop of phenolphthalein solution as the indicator. Repeat the above procedure with the second fraction. If the second fraction requires less than 0.10 milliliter of the 0.015 *N* NaOH solution and all the acetic acid has been distilled over, the determination is completed. If greater than this, collect a third fraction of approximately 10 milliliters and titrate this as before. Total volumes of NaOH used and calculate results as follows:

$$(\text{Milliliters of NaOH} \times N \text{ NaOH} \times 0.043 \times 100)/\text{Weight sample in grams} = \text{Percent acetyl.}$$

(7) *Crystallinity.* Proceed as directed in § 440.80a(b)(5)(iii) of this chapter.

§ 436.516 Tetracycline-neomycin complex powder topical; tetracycline hydrochloride-neomycin sulfate powder topical.

(a) *Potency*—(1) *Tetracycline-neomycin complex powder*—(i) *Tetracycline content.* Proceed as directed in § 436.514(a)(2), except use water in lieu of 0.1 *N* HCl for dissolving the sample. Its tetracycline content is satisfactory if it contains not less than 85 percent of the equivalent number of milligrams of tetracycline hydrochloride that it is represented to contain.

(ii) *Neomycin content.* Using 0.1 *M* potassium phosphate buffer, pH 8.0, dilute an appropriate aliquot of the aqueous solution, prepared as directed in paragraph (a)(1) of this section, to a final concentration of 1 μg. per milliliter